Purification and properties of human erythrocyte arginase

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SUMMARY. An efficient method for purification of human erythrocyte arginase was developed. This method included two new procedures, hydrophobic chromatography and immunoaffinity chromatography, and yielded 0·7 mg of homogeneous arginase protein from 2·1 L of haemolysate. The molecular weight of native arginase was estimated to be 105 000 by gel filtration on a Sephadex G-150 column, and that of its subunit 35 000 by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate. This indicates that the native enzyme is composed of three homologous subunits. Amino acid composition of human erythrocyte arginase was found to be very similar to that of liver arginase of several other mammals. After dialysis against distilled water, the purified arginase still retained its enzymatic activity which was decreased by EDTA and reversibly restored by Mn(II) ion. A specific polyclonal antibody for use in an immunoassay was also produced. This antibody revealed one single band on immunoelectrophoretic analysis of the acetone powder extract, suggesting absence of arginase isoenzymes in human erythrocytes.

Arginase (EC 3.5.3.1; L-arginine amidinohydrolase) is one of the urea cycle enzymes. The liver contains a large amount of arginase.[1] Therefore, liver damage by agents such as carbon tetrachloride result in appearance of elevated blood arginase activity in the test animals,[2] and arginasemia[3] has been reported to be a useful index for diagnosis of hepatic disorders.[1] However, a routine assay for arginase activity in the presence of high concentrations of urea was not possible because the assay of arginase was dependent on the rate of formation of urea from substrate L-arginine. Perhaps for that reason, the potential usefulness of arginasemia as a diagnostic index has been overlooked, compared to other hepatic enzymes in the serum such as glutamate-oxaloacetate aminotransferase, glutamate-pyruvate aminotransferase, lactate dehydrogenase, etc. These latter enzymes are now assayed routinely, but they should be much less organ-specific than liver arginase. We became interested in establishing an immunoassay method for arginase, which could be used for analysis of serum without being influenced by urea present at a high concentration in the sample. For that purpose, we needed to obtain a sufficient amount of highly purified arginase as the antigen.

We chose human erythrocyte as the starting material for the purification of arginase. Erythrocyte contains arginase which is immunologically cross-reactive with liver arginase.[4-7] Purification of human erythrocyte arginase was reported by Nishibe[6] and by Berüter *et al.*,[7] but the yield was very low or the criteria for product purity were inadequate. We developed an efficient method for purification, which included two new procedures, hydrophobic chromatography and immunoaffinity chromatography. The yield was up to 0·7 mg of homogeneous arginase protein from 2·1 L of haemolysate. We have also studied some properties of the purified enzyme, and succeeded in raising a specific antibody to be used for the immunoassay.

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Materials and methods

MATERIALS

CM Sephadex C-50, Sephadex G-100 and G-150, and CNBr-activated Sepharose 4B were obtained from Pharmacia, Sweden. DEAE-cellulose

(DE52) was obtained from Whatman, UK, and TSK-GEL Butyl-Toyopearl 650M, from Toso Co., Tokyo, Japan. Molecular weight standards were obtained from Sigma Chemical Co., St. Louis, USA. Other reagents were obtained from the following commercial sources: urease (type II) and glutamate dehydrogenase (GLDH)(type II) from TOYOBO Co., Osaka, Japan; β-nicotinamide adenine dinucleotide phosphate tetrasodium salt, reduced form (NADPH), from Kohjin Co., Tokyo, Japan; anti-human immunoglobulin (IgG, A and M mixture) goat antibodies from Capell Co., USA.

ARGINASE ACTIVITY

Arginase activity was determined by measuring the amount of urea produced by arginase from the substrate,[8] L-arginine, using coupled reactions catalyzed by urease and GLDH. Each assay mixture had a final volume of 2·0 mL and contained 0·1 M Tris–HCl buffer, 50 mM L-arginine, 5 mM α-ketoglutarate, 0·25 mM NADPH, 60 units of urease and 28 units of GLDH at pH 8·3. The enzymatic reaction was initiated by adding 5 μL of the sample to be tested to 2·0 mL of the above mixture. After incubation for 1 min at 30°C, NADPH depletion was monitored by following the linear decrease in absorbance at 340 nm. One unit of arginase was defined as the amount of enzyme that released 1·0 μmol of urea for 1 min under the conditions employed.

ELECTROPHORESIS

Polyacrylamide gel (12·5%) electrophoresis in the presence of 0·1% sodium dodecyl sulphate (SDS-PAGE) was carried out according to the method of Laemmli.[9] An ATTO (Osaka, Japan) electrophoresis apparatus was used. All gels were stained with Coomassie brilliant blue.[10] For immunoelectrophoretic blot analysis, proteins were first subjected to SDS-PAGE, and transferred from the gels to a nitrocellulose membrane by the method of Towbin *et al.*[11] Proteins on the membrane were reacted first with anti-arginase antibody and then with horse radish peroxidase-conjugated goat anti-rabbit IgG (Capell, USA). o-Dianisidine was used as substrate for the colour development, according to the method of Hawkes *et al.*[12]

AFFINITY COLUMNS

Immunoaffinity-Sepharose was prepared from CNBr-activated Sepharose 4B by the method of Brusdeilins *et al.*[13] Anti-human immunoglobulin goat antibody (2 mg) was dissolved in 2·0 mL of 0·1 M $NaHCO_3$ buffer, pH 8·3, containing 0·5 M NaCl (coupling buffer) and dialysed against the same buffer. CNBr-activated Sepharose 4B (0·5 g of dry gel) was reswollen in 200 mL of 1 mM HCl per g of gel, washed with coupling buffer, and immediately transferred to coupling buffer containing the immunoglobulins. After coupling overnight at 4°C, the gel was washed once in coupling buffer, and the remaining active groups were blocked with 1 M Tris–HCl buffer, pH 8·3, for 2 h at room temperature. The gel was washed alternately twice with coupling buffer and 0·1 M acetate buffer, pH 4·0, containing 0·5 M NaCl, and then stored in 10 mM sodium phosphate buffer, pH 7·2, containing 0·15 M NaCl and 0·01% sodium azide. For chromatography, 3 mL of the gel was packed into a 9 × 80 mm column. This column was called column A.

The arginase-Sepharose column was prepared by coupling purified arginase after step 6 (see Table 1) (approximately 1 mg of protein) to CNBr-activated Sepharose 4B(0·2 g of dry gel) in

TABLE 1. Purification of human erythrocyte arginase

Step*	Volume (ml)	Total protein (mg)	Specific activity (U/mg)	Purification (fold)	Total activity (U)	Yield (%)
1	2100	731000	0·00116	1	847	100
2	590	2290	0·766	661	1750	207
3	590	450	3·46	2980	1560	184
4	2·8	38·6	26·5	22900	1030	121
5	2·0	11·7	33·8	29200	396	47
6	7·5	0·70	204	176000	143	17

* Steps are: 1, haemolysate; 2, extraction from acetone powder, followed by heat treatment at 60°C and pH 8·8 for 10 min; 3, CM Sephadex C-50 chromatography; 4, gel filtration on Sephadex G-100; 5, hydrophobic chromatography on TSK-GEL Butyl-Toyopearl 650M; and 6, immunoaffinity chromatography on columns.

the same way as for column A. A 9 × 20 mm column of the gel was prepared and called column B.

ANTIBODY AGAINST HUMAN ERYTHROCYTE ARGINASE

Japanese albino male rabbits, 6 months old, were immunised with partially purified arginase (step 4, see Table 1; approximately 1·5 mg protein/kg body weight). Antiserum was obtained one week after the first booster immunization. After ammonium sulfate (60%) precipitation from the antiserum, partially purified antibody was obtained by DEAE-cellulose chromatography. It was further purified using column B, yielding approximately 0·8 mg of specific antibody.

AMINO ACID COMPOSITION

A JEOL JLC-300 HPLC apparatus equipped with a 6·0 × 90 mm column of LC-R-6 gel (JEOL, Tokyo, Japan) was used. Purified arginase (approximately 0·4 mg), desalted and lyophilised, was hydrolysed in 0·3 mL of 6 N HCl for 24 h at 110°C under reduced pressure. The hydrolysate was mixed with 0·5 mL of 0·2 M sodium citrate buffer, pH 2·2. A 100 μL aliquot of the mixture was subjected to the analysis.

PROTEIN DETERMINATION

Protein concentration was determined by the method of Lowry *et al.*[14] using bovine serum albumin as the standard.

Results

PURIFICATION OF HUMAN ERYTHROCYTE ARGINASE

The purification procedure is summarised in Table 1. Up to step 4, the method of Nishibe[6] was employed. Further purification was carried out at 4°C as follows.

Hydrophobic chromatography (step 5)

The product obtained after the gel filtration was dissolved in 20 mL of 10 mM Tris-HCl buffer, pH 7·4, containing 3·2 M NaCl. The sample was applied to a column filled with TSK-GEL Butyl-Toyopearl 650 M previously equilibrated with the same buffer. After washing the column well with the same buffer, proteins were eluted with a linear reverse gradient of NaCl from 3·2 to 0 M. The enzyme could be resolved into two peaks (Fig. 1a). The first peak fractions (Nos 46–52) were pooled and dialyzed against 10 mM Tris-HCl buffer, pH 7·4.

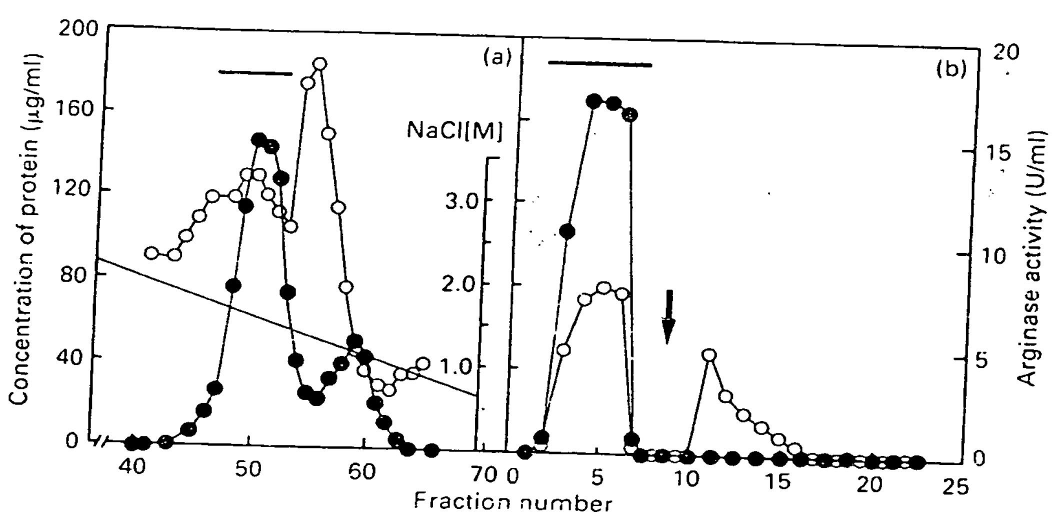

FIG. 1. Purification of human erythrocyte arginase by hydrophobic chromatography (a) and immunoaffinity chromatography (b). In (a), hydrophobic chromatography of human erythrocyte arginase after Sephadex G-100 gel filtration, step 4 enzyme (38·6 mg, 2·8 mL) was applied to a TSK-GEL Toyopearl 650M column (27 × 100 mm) previously equilibrated with 10 mM Tris-HCl buffer, pH 7·4, containing 3·2 M NaCl. After washing with the same buffer, proteins were eluted with a linear decreasing gradient of NaCl from 3·2 to 0 M. Fractions of 3·0 mL were collected. The first peak fractions (Nos 46–52), indicated by a horizontal line, were pooled and dialyzed against the starting buffer, containing 1 mM Mn(II) ions, and concentrated. ○, proteins eluted; ●, arginase activity. In (b), step 5 enzyme (11·7 mg, 2·0 mL) was applied to column A (9 × 80 mm) previously equilibrated with 10 mM Tris-HCl buffer, pH 7·4, containing 1 mM Mn(II). The column was washed with 10 ml of the same buffer, and then immunoglobulins were eluted with 0·1 M glycine–HCl buffer, pH 2·3, as shown by a vertical arrow. Fractions of 2·0 mL were collected. The solutions containing the enzyme, indicated by a horizontal line, were pooled, dialysed, and stored until use at −80°C after adding Mn(II) ions to obtain a final concentration of 0·5 mM. ○, proteins eluted; ●, arginase activity.

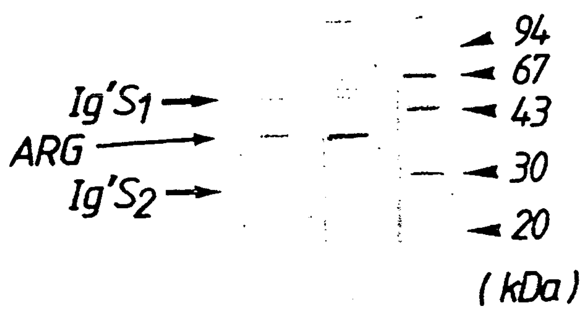

FIG. 2. SDS-polyacrylamide gel electrophoresis of human erythrocyte arginase. The enzyme before (lane 1, approximately 10 μg) and after step 6 (lane 2, approximately 20 μg) was run on a gel for 1·5 h at room temperature. Proteins were stained with Coomassie brilliant blue. $Ig'S_1$ and $Ig'S_2$ in the figure indicate the migration positions of heavy and light chains, respectively, of immunoglobulins. ARG is for a position of subunit of erythrocyte arginase. Lane M is for marker proteins: phosphorylase b (M_r 94 000), bovine serum albumin (67 000), ovalbumin (43 000), carbonic anhydrase (30 000), soybean trypsin inhibitor (20 100) and α-lactalbumin (14 400).

Immunoaffinity chromatography (step 6)
Since SDS-PAGE of the proteins eluted at step 5 showed only arginase and immunoglobulins as major bands (Fig. 2, lane 1), we used column A for the removal of immunoglobulin. The eluant from this column was collected, dialysed against distilled water, and lyophilised. SDS-PAGE of the enzyme after this step (Fig. 2, lane 2) shows essential homogeneity of the product.

PROPERTIES OF HUMAN ERYTHROCYTE ARGINASE

Molecular weight
The molecular weight of native arginase, estimated by gel filtration on a Sephadex G-150 column, was 105 000 (Fig. 3a), and that of the subunit of the enzyme, estimated by SDS-PAGE, was 35 000 (Fig. 3b). The data suggest that the native enzyme is composed of three homologous subunits.

Effect of EDTA and Mn (II)
As shown in Fig. 4, a loss of enzyme activity was observed in three different buffer solutions containing no EDTA. The loss was considerably accelerated by adding EDTA to these buffer solutions. However, the enzyme regained its catalytic activity by the addition of Mn(II) ions 49 h after the addition of EDTA, indicating that arginase activity is dependent on Mn(II) ions, and that the binding between arginase and Mn(II) is reversible.

Effects of arginine analogues
K_i values and the mode of inhibition determined by the Lineweaver–Burk plots are given in Table 2. Under the conditions employed, the value of K_m for L-arginine was found to be 1·6 mM. The following derivatives of arginine showed no inhibition: H-Arg-OMe, Acetyl-Arg-OH, benzyloxycarbonyl-Ala-Arg-OH, H-Gly-Arg-OH, butoxy-

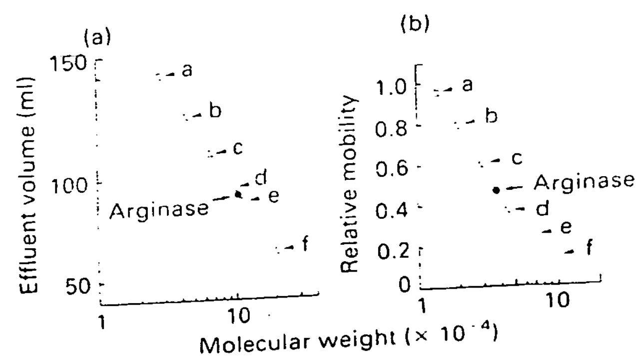

FIG. 3. Determination of the molecular weight of human erythrocyte arginase by gel filtration with Sephadex G-150 (a) and its subunit by SDS-PAGE (b). In (a), purified enzyme (0·2 mg) and standards (3 mg each) were used. Marker proteins (○): a, carbonic anhydrase (M_r, 30 000); b, ovalbumin (43 000); c, bovine serum albumin (67 000); d, phosphorylase b (94 000); e, β-galactosidase (116 000); f, myosin (205 000). ●, human erythrocyte arginase. In (b), approximately 20 μg of purified enzyme was used. Marker proteins (○): a, α-lactalbumin (M_r 14 400); b, soybean trypsin inhibitor (20 100); c, carbonic anhydrase (30 000); d, ovalbumin (43 000); e, albumin (67 000); f, phosphorylase b (94 000). ●, human erythrocyte arginase.

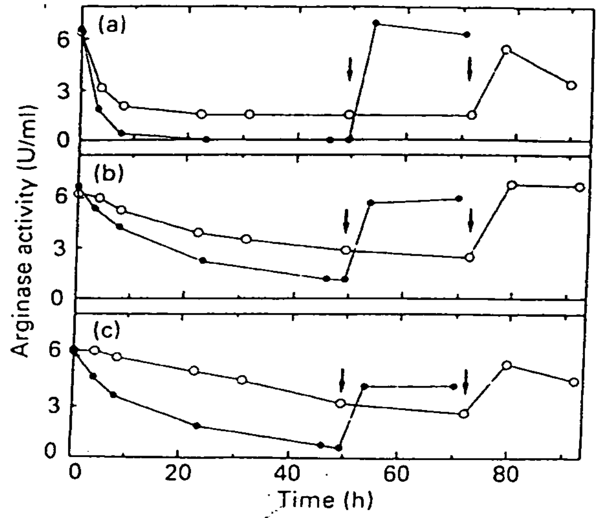

FIG. 4. Effect of EDTA and Mn(II) ions on human erythrocyte arginase and its reactivation with Mn(II) ions. Purified enzyme (10 μg/ml) was allowed to stand at 25°C in the presence (●) and absence (○) of 2 mM EDTA for 49 h and 73 h, respectively, after which time 5 mM Mn(II) was added (arrows). Buffers used are: 0·1 M sodium phosphate buffer, pH 7·0 (a); and 0·1 M glycine-NaOH buffer, pH 8·3 (b) and 10·0 (c). Aliquots were withdrawn at given time intervals, and arginase activities were determined without further addition of Mn(II) ions to the reaction mixture.

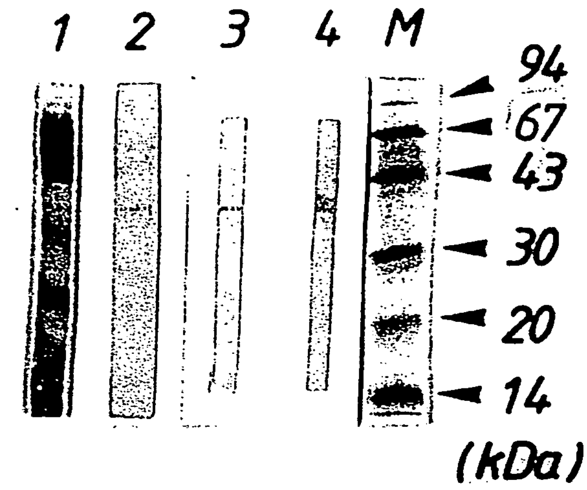

FIG. 5. Immunoelectrophoretic blot analysis of human erythrocyte arginase with specific antibody. Arginase-specific antibody, purified using column B, was employed for immunostaining. Lanes 1 and 2, preparation before heat treatment in step 2 (100 μg protein); lane 3, preparation after step 4 (10 μg protein); lane 4, preparation after step 6 (10 μg protein). Lane M is for marker proteins, as in Fig. 2. For lanes 1 and M, proteins were stained with Coomassie brilliant blue. Lanes 2, 3 and 4 were immunostained with purified anti-human erythrocyte arginase subunit IgG.

carbonyl-Val-Leu-Gly-Arg-OH, H-Gly-Gly-Arg-OH, N-α-acetyl-Leu-Leu-Arg-H.

Amino acid composition

The amino acid composition found for human erythrocyte arginase was compared with data previously reported.[15-18] Only data for liver arginase were available, and this was recalculated in terms of mol% for comparison. We did not determine half-cystine or tryptophan, but this is unlikely to have a significant effect on the composition, assuming that the low content of these amino acids in liver arginase is paralleled in the erythrocyte enzyme. The data shown in Table 3 indicate a remarkable similarity between arginases of human and other mammals, while they differ significantly from chicken liver arginase.

ARGINASE-SPECIFIC ANTIBODY

The results of immunoelectrophoretic blot analysis are shown in Fig. 5. The arginase preparation purified to the final step 6 gave a single and strongly immunostained band (lane 4), whose molecular mass was 35 kDa, an identical value to that found by protein staining on SDS-PAGE (Fig. 2).

Preparations after steps 2 and 4 (lanes 2 and 3) also gave only one band each, but staining intensity was much less than that for lane 4, indicating effective enrichment of arginase pro-

TABLE 2. Inhibition of human erythrocyte arginase by arginine analogs

Arginine analogue	Concentration (mM)	Mode of inhibition	K_i (mM)
Lysine	10	competitive	2·7
Homoarginine	5	competitive	5·0
Argininosuccinic acid	5	non competitive	22·5
Canavanine	5	non competitive	34·1
Octopine	5	non competitive	36·4

TABLE 3. Amino acid composition of arginase*

Amino acids	Human erythrocyte arginase	Liver arginase			
		Pig[15]	Bovine[16]	Rat[17]	Chicken[18]
Lysine	7·5	9·0	8·2	9·0	5·5
Histidine	2·8	3·3	2·8	2·4	1·7
Arginine	4·0	4·4	3·8	3·4	5·2
Aspartic acid	9·2	9·4	8·7	9·0	10·5
Threonine	6·6	5·9	5·6	7·1	4·6
Serine	6·0	5·3	4·5	6·2	5·6
Glutamic acid	8·1	8·0	8·9	9·0	13·7
Proline	7·5	6·7	6·7	7·4	5·2
Glycine	11·9	11·5	10·1	9·3	8·2
Alanine	5·5	5·5	5·6	6·7	8·2
Half-cystine	ND	ND	1·7	0·7	2·0
Valine	7·4	8·6	8·4	8·9	7·4
Methionine	0·9	1·7	1·4	1·3	1·7
Isoleucine	6·4	4·7	6·0	5·3	4·3
Leucine	10·5	8·5	10·0	7·4	8·9
Tyrosine	2·7	3·3	3·2	2·5	2·6
Phenylalanine	2·9	4·4	3·4	3·2	3·3
Tryptophan	ND	ND	0·9	1·3	1·2

* Values are in mol%. For liver arginase, reference numbers are shown. ND, not determined and omitted from the calculation of mol% values.

tein as the purification steps proceeded. It should be noted that the affinity-purified antibody used was sensitive enough to reveal the presence of arginase in the crude extract from the acetone powder, and that even at that stage only one molecular species of arginase (subunit protein) could be detected.

Discussion

Several methods for the purification of human erythrocyte arginase have been reported.[6, 7] Perhaps the best preparation was obtained by Nishibe,[6] who extracted the enzyme from agar gels after electrophoresis. However, the latter procedure minimised the yield, with reduced specific activity. The modified method reported in this paper permitted us to obtain a large amount of the purified enzyme (Table 1). The difference in hydrophobic properties between arginase and other proteins was utilised effectively to isolate the enzyme from other proteins (Fig. 1a). The use of Sepharose-immobilised immunoglobulins was also effective in separating arginase from contaminating immunoglobulins (Figs 1b and 2), which otherwise would be difficult to remove.

Several workers[17, 19] have reported that arginase is composed of four homologous subunits. However, the present study demonstrated that the molecular weight of native erythrocyte arginase was estimated to be 105 000 by gel filtration on a Sephadex G-150 and that of a subunit of the enzyme 35 000 by SDS-PAGE (Fig. 3). We conclude that erythrocyte arginase is composed of three identical subunits. The catalytic activity of rat liver arginase depends to a large degree on the presence of Mn(II) ions. During certain purification steps, eg column chromatography and dialysis, part of the metal ion is lost, and the specific activity drops gradually.[20] Vielle-Breitburd and Orth[21] reported that when the tightly bound ions were removed from rabbit liver arginase, irreversible inactivation occurred. In this study, the effective removal of the metal ion from arginase-manganese complex (holoenzyme) by 5 mM EDTA resulted in a considerable loss of arginase activity compared with that of the non-treated enzyme at three different pH values. However, the arginase activity was restored by adding Mn(II) ions, suggesting reversible formation of the holoenzyme (Fig. 4).

Partially purified arginase from human erythrocyte was used for raising polyclonal antibody in rabbits, which was further affinity-purified with the highly purified arginase preparation. The final product IgG was shown to be monospecific for arginase protein even when the crude extract from acetone powder was used for the Western blot analysis (Fig. 5). The latter result provides us two important lines of information. Firstly, the purified antibody has sufficiently high sensitivity for use in immunoassay in the clinical laboratory.

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Secondly, the finding of a single band is evidence for the existence of one major, or perhaps only one molecular species of arginase in the human erythrocyte, in contrast to reports of various arginase isoenzymes by other workers.[22-26] The appearance of a single peak on CM Sephadex chromatography (Fig. 1a) and on a Sephadex G-150 column (Fig. 3a), together with the reasonably high yield (17%) of the purified enzyme (Table 1), implies that isoenzymes different from the major one purified, (if any) are present in low concentrations. The apparent polymorphism of arginase molecules could be caused either by self-association[15, 27] or by proteolytic modification during the purification procedures. Recently, a single cDNA for human liver arginase was cloned, and this was used for constructing a plasmid which could be expressed in *Escherichia coli*.[28] The identity of the product from *E. coli* with human erythrocyte arginase obtained in the present study has been confirmed (M Ikemoto *et al.*, unpublished observations) supporting the idea that most, if not all, of the previously reported arginase isoenzymes must have represented artifacts.

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